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Modification of pathways with *Pseudomonas* for the extraction and subsequent conversion of algae to butanol

By Danielle Frechette

Department of Biological and Agricultural Engineering, University of Arkansas, Fayetteville,
AR 72701

Research Advisor: **Dr. Jamie Hestekin**

Department of Chemical Engineering, University of Arkansas, Fayetteville, AR 72701



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ABSTRACT

As the demand for alternative fuels steadily increases, algae continue to be an excellent source for the development of biofuels. While algae have exhibited substantial potential for butanol production, there are limitations when it comes to the extraction process—its inability to withdraw oils and sugars usable for fermentation from cell walls. The genes of two bacteria were combined, creating a new organism that can both extract sugars and oils from algal cell walls and create butanol, simplifying the fermentation process while increasing efficiency. *Pseudomonas flourescens*, an obligate aerobe, has been shown in literature to degrade these cell walls to make sugars and oils available for fermentation. An anaerobic bacterium, *Clostridium acetobutylicum*, is commonly used for biofuel production because its ability to make biobutanol, among other biofuels. *P. flourescens* is absent one gene, alcohol dehydrogenase (AdhE2), from *C. acetobutylicum*'s biobutanol pathway. The AdhE2 gene was extracted from *C. acetobutylicum* using PCR and then cloned it into *P. flourescens* using the pCN51 shuttle vector. The newly created organism that can both create biobutanol and perform algae lysis was cultured in glucose rich nutrient broth. Biobutanol production was confirmed using gas chromatography and HPLC.

1. INTRODUCTION

1.1 Need for Alternative Fuel

It is increasingly becoming apparent that our society and environment is in need of an alternative fuel source that is both efficient and affordable. The excessive use of coal, gasoline, and oil is damaging to our surroundings and the health of the public, and the cost of fuel is becoming too expensive for many to afford. There are multiple on-going research projects that are in the process of developing efficient methods for creating biofuel (1-3); this fuel could potentially be integrated into society's everyday lifestyle. Not only is reducing the global emissions of greenhouse gases a concern for many researchers, but with the recent recession, an economical fuel is also ideal. There is a set of criteria that an alternative fuel source should meet. This includes an increase in net energy compared to the energy sources used to produce it, the ability to be produced in mass quantities, a decrease in production costs relative to the fuel it is replacing, the energy source not competing with our food supply, and also a decrease in negative environmental impacts. While there are several crops among other fossil fuels available to use as a fuel source, along with many existing and novel methods for producing biofuels, fermentation via algae seems to potentially be the superlative in fitting the economical and eco-friendly profile.

1.2 Algae as a Fuel Source

Within the past few years, researchers have become more interested in using algae as an energy source. The more we learn about the benefits of this microorganism as the source for biofuels, the more attractive it becomes because the advantages over other crops and fossil fuels are abundant. Algae have the potential for the largest production levels of biofuel, partly due to

the fact that they have the fastest life cycle of any existing microorganism. Some species of algae have a doubling time of just a few hours; this is much higher than the life cycle of any land plant (4). Their efficiency in sunlight uptake is superior to most other plants and algae possess the ability to grow in a wider range of sunlight intensities, allowing for flexibility in geographical areas for production and the duration of hours per day algae are able to grow. There are also over 300,000 strains of algae, which is far greater than any variety of crops (4) and allows for diversity when it comes to biofuel production via different biomasses. In comparing aquatic microbial oxygenic photoautotrophs, which includes algae, to crops commonly used as a fuel source, the photoautotrophs' production levels are far superior. Dismukes *et al.* reported a 5.4-10 fold greater production of annual biomass yield (in dry metric tons/ha×yr) compared to hybrid strains of corn grain, and a 2.5-10 fold greater production compared to switch grass. With these values converted to raw energy content (GJ/ha×yr), the photoautotrophs had approximately a 6-12 fold increase in energy over conventional crops (5). This stands as a significant advantage in using algae as a fuel source.

Algae can be grown on a minimal amount of land, which can be of low quality, eliminating the concern of space and costs to maintain healthy living conditions for growing biomass. Algae can survive in diverse, sometimes unfavorable, conditions and also have the ability to grow year round. While it is necessary to provide conventional biomass with proper nutrients that is often costly, algae have the ability to extract their nutrients from even wastewater, making maintenance minimal and upkeep relatively easy. This attribute allows for recycling our water while providing algae with the necessary nutrients to grow, at no extra cost. The production of crops does not allow this opportunity.

Algae use a significant amount of nitrogen, carbon dioxide, and phosphorus, which can be removed from their water source and cleanses the environment. This produces negative greenhouse emissions in the process of growing, harvesting, refining, and burning of the fuel, again, contributing to cleaning the environment. Groom *et al.* reported that algae can potentially produce -183 kg CO₂/MJ of greenhouse gas emissions, while the more common ethanol production via various grasses produces +81-58 kg CO₂/MJ. Groom *et al.* also reported that the percent of US crop land needed to meet half of the transportation fuel demands is 1.1-1.7% for algae, 46-57% for corn, and 157-262% for grasses to produce ethanol (6). Algae stand as the best option when it comes to a fuel source.

1.3 Butanol's Superiority

Algal fermentation can result in multiple byproducts, including ethanol, butanol, and acetone. This study focuses on the production of butanol because it is potentially the most efficient fuel additive today's society could be using. Butanol has a relatively high energy content, providing 105,000 BTU per gallon (7), while ethanol, the fuel that accounts for 99% of biofuel used in the US (8), only provides approximately 77,000 BTU per gallon (9). The conversion from gasoline to butanol would be relatively simple because butanol can replace gasoline 1:1 due to its similar energy content. It can be mixed with gasoline at any ratio or even replace gasoline completely, and it has the ability to withstand water contamination because of its hydrophobicity. Butanol is safer to use and less corrosive than other alternative fuels, resulting in safe transportation, handling, and use under extreme temperatures, and it can be transported with existing infrastructure. The issue with using butanol as an alternative fuel is that it uneconomical using most current production methods, but there are methods that can be used, such as fermentation via algae, which serve as less expensive solution.

1.4 Goal of Project

Butanol fermentation via algae is typically viewed as a tedious process, due to the multiple steps that are necessary to 1) extract the carbohydrates and sugars from the algal cells and 2) control the fermentation process. Having the extraction and fermentation in two major steps is often where time serves as a great inefficiency in the process. There are existing bacteria that can perform either step one or two, but none that can do the entire process alone. By creating a bacteria that will do both-create butanol and extract sugars and carbohydrates from algal cell walls, one can essentially eliminate the “multistep” fermentation process and merge it into one, efficient method.

The goal of this study was to combine the genes of *P. fluorescens* and *C. acetobutylicum* to create one bacterium that could convert algae to biobutanol on its own. As far as we know, there are no existing studies that have created a bacterium that have this ability. *P. fluorescens* is an obligate aerobe that has the ability to lyse the cell walls of algae and abstract the sugars and carbohydrates from within, making them available for fermentation. *C. acetobutylicum* is an obligate anaerobe that is commonly used in fermentation and can produce multiple products, including acetone, ethanol, and butanol. By removing the necessary genes from *C. acetobutylicum* and inserting them into a shuttle vector which could be cloned into *P. fluorescens*, we could theoretically create a synthetic pathway in *P. fluorescens* to produce biobutanol.

1.5 Similar Work

Ingram *et al.* did similar work by demonstrating an increase of ethanol production by the insertion of the necessary genes from *Zymomonas mobilis* into *Escherichia coli*. By expressing high levels of alcohol dehydrogenase II and pyruvate decarboxylase in *E. coli*, they were

successful in observing an increase in biofuel production. They suggested that the insertion of the necessary genes, such as these, into other bacteria, can alter their fermentation products (10). Atsumi *et al.* also published work in which they genetically engineered *E. coli*, but they did by inserting the necessary genes from *C. acetobutylicum* in order to get 1-butanol production from *E. coli*. This was done as a means towards creating a “user-friendly organism” that was well studied and could result in optimal production (11). This study was very similar to ours in that the genes were cloned from the same bacterium in order to get 1-butanol production as a fermentation byproduct. Atsumi *et al.* were successful in fermenting biobutanol from *E. coli*. This study suggests we would be successful in genetically engineering *P. fluorescens* to achieve biobutanol production.

2. MATERIALS AND METHODS

2.1 Cell Culture

P. fluorescens was cultured in 10g SIGMA Luria-Bertani (LB) broth per 500 mL distilled water at 37°C. Culture plates were made with 7.5 g Agar (Difco)/10 g LB/500 mL DD water. *E. Coli* containing pCN51 ATCC 77100 was cultured using recommended medium from ATCC containing LB broth and 25 ug/mL kanamycin at 37°C. *C. acetobutylicum* ATCC 824 was cultured anaerobically in medium consisting of 3.5 mg/L yeast extract (Amresco), 20 mg/L glucose monohydrate (EMD), and 6.5 g/L tryptone (Mobio Laboratories).

2.2 Electrophoresis

Gels were composed of 60 mL Tris-Acetate- EDTA (TAE) at 1x, 5 uL ethidium bromide, and 0.8g Agarose. Electrophoresis was run for 30 minutes at 100v and 400mA.

2.3 Plasmid Isolation

pCN51 ATCC 77100 was extracted from *E. coli* using the QIAprep Spin Miniprep Kit (QIAGEN). Prior to start of protocol, 1mL of cell suspension was centrifuged for 30-60 seconds at 1000 x g and supernatant was disposed of. The process was repeated until no medium remained. To confirm isolation, the DNA was observed in an ethidium bromide-stained gel under UV light. Plasmid was stored at -20°C.

2.4 Plasmid Amplification

pCN51 was amplified in *P. fluorescens* with electroporation using a modified version of Gene Bridges – Quick and Easy *E. coli* Gene Deletion Kit, Version 2.0 (January 2007) Section 6.2 “Transformation with Red/ET expression plasmid pRedET”. Protocol was followed beginning at step 3 and preparing cells for electroporation. 1 ul of pCN51 at concentration 42.8 ng/uL was transferred to chilled cuvette. Electroporated cells were incubated and shaken at 37°C for 60 min in 300 ul LB broth. Cells were transferred to 5 ml LB broth + 5 ul kanamycin medium for growth.

2.5 Enzyme Digestions

Gels for enzyme digestions were composed of 100 mL TAE buffer (1x), 0.8g Agarose, and 5 uL ethidium bromide. Gel electrophoresis was run at 100 V and 400 mA for approximately 30 minutes. Enzyme solutions were mixed with dye for an approximate target of 6x total solution/dye. A 1kB DNA ladder at 6x concentrate was used for comparison. *XbaI/BamHI*: 2 uL sterile water, 8 uL pCN51 DNA, 1.5 uL 10x BSA, 1.5 uL buffer (10x), 2 uL enzyme; *SphI*: 8 uL pCN51 DNA, 1 uL buffer (10x), 1uL enzyme; *NcoI*: 1 ul pCN51 DNA, 1 ul NEBuffer3 (10x), 1 ul enzyme; *BspHI*: 8 ul pCN51 DNA, 1 ul NEBuffer 4 (10x), 1 ul enzyme; *HindIII*: 8 ul pCN51

DNA, 1 ul NEBuffer 2 (10x), 1 ul enzyme; *Sma*I: 8 ul pCN51 DNA, 1 ul NEBuffer 4 (10x), 1 ul enzyme. Digestions were left in a 37°C water bath for approximately 1 hour before performing electrophoresis.

2.6 PCR

To remove GFP from pGFPuv, the following primers were used: forward primer TCACTCATTAGGCACCCCAGGC and reverse primer ACCGAAACGCGCGAGACGAA. The portion of the plasmid extracted was approximately 1 kb in length and consisted of the 5' multiple cloning site, the lac operon, and the GFP protein. PCR protocol, Platinum[®] PCR SuperMix (Invitrogen), was used with 45 uL of Platinum[®] PCR SuperMix as the polymerase, 0.5 uL of both forward and reverse primers, and 1 uL template DNA solution (pGFPuv). Run time was 2 hours and 12 minutes. PCR was confirmed with gel electrophoresis.

3. RESULTS AND DISCUSSION

3.1 1-butanol Pathway

Atsumi *et al.* published the 1-butanol production pathway of *C. acetobutylicum*, showing the genes that contributed to the 1-butanol fuel production (Fig 1).

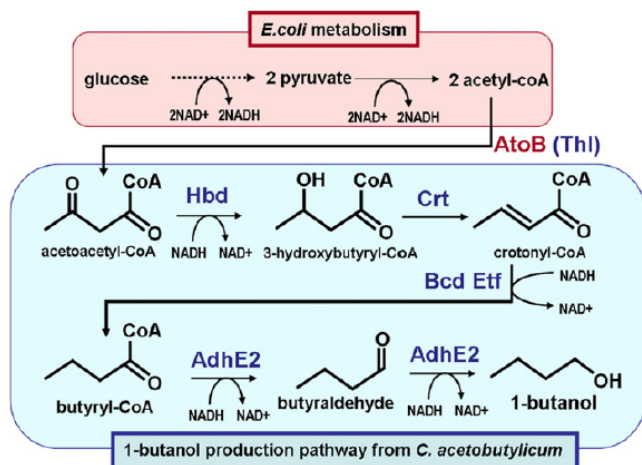


Figure 1. “Schematic representation of 1-butanol production in engineered *E. coli*. The engineered 1-butanol production pathway consists of six enzymatic steps from acetyl-CoA. *AtoB*, acetyl-CoA acetyltransferase; *Thl*, acetoacetyl-CoA thiolase; *Hbd*, 3-hydroxybutyryl-Co A dehydrogenase; *Crt*, crotonase; *Bcd*, butyryl-CoA dehydrogenase; *Etf*, electron transfer flavoprotein; *AdhE2*, aldehyde/alcohol dehydrogenase.” (11)

By using this information and identifying these genes in the genome of *P. fluorescens* by using online databases, we were able to conclude that *P. fluorescens* was absent just one gene from this pathway: aldehyde/alcohol dehydrogenase 2, *AdhE2* (Table 1). By the insertion of *AdhE2*, *P. fluorescens* should be able to have the required genes to code for the enzymes to produce biobutanol.

Table 1	
Comparison of <i>C. acetobutylicum</i> 1-butanol production pathway to <i>P. fluorescens</i>	
Genes present in <i>C. acetobutylicum</i> 1-biobutanol production pathway	Genes present in <i>P. fluorescens</i>?
<i>Thl</i> , acetoacetyl-CoA thiolase	yes
<i>Hbd</i> , 3-hydroxybutyryl-CoA dehydrogenase	yes
<i>Crt</i> , crotonase	yes
<i>Bcd</i> , butyryl-CoA dehydrogenase	yes
<i>Etf</i> , electron transfer flavoprotein	yes
<i>AdhE2</i> , aldehyde/alcohol dehydrogenase	no

3.2 Shuttle Vector

The shuttle vector chosen to clone into *P. fluorescens* was one derived from a 10-kb plasmid of *Pseudomonas savastanoi*, pPS10. Nieto *et al.* created multiple cloning vectors specifically for the use of genetic manipulation and cloning into various strains of *Pseudomonas* (12). Plasmid pCN51 was chosen from these various shuttle vectors for its smaller size (5.9kb)

and ability to be transformed into both *Pseudomonas* and *E. coli* strains (Fig 2). The published vector map showed kanamycin resistance and four restriction enzyme sites.

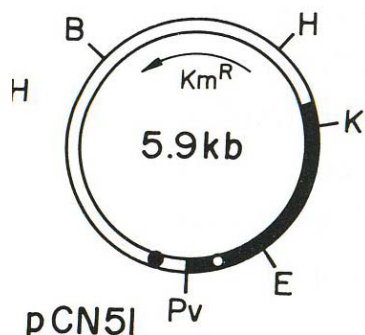


Figure 2. Vector map of pCN51 shuttle vector, derived from pPS10 and published by Nieto *et al.* The plasmid contains kanamycin resistance and shows four restriction enzyme sites: B, *Bam*HI; H, *Hind*III; K, *Kpn*I; Pv, *PVU*II. pCN51 resulted from a spontaneous deletion of a 2.6kb fragment from pCN50.

3.3 Restriction Mapping

There was little information reported on pCN51. Due to the fact that the plasmid is not sequenced, it was necessary to do restriction digests to locate other restriction enzyme sites (Table 2). These were used as possible sites for the insertion of *AdhE2* and for amplification in *Pseudomonas*.

Table 2

Number of Restriction Enzyme Cuts in pCN51 Shuttle Vector		
Restriction Enzyme	0 Cut Sites	1 Cut Site
<i>Sph</i> I		✓
<i>Hind</i> III		✓
<i>Xba</i> I	✓	
<i>Bam</i> HI		✓
<i>Nco</i> I		✓
<i>Bsp</i> HI		✓
<i>Bso</i> BI		✓
<i>Sma</i> I		✓

The following gels show the results from the electrophoresis and restriction mapping.

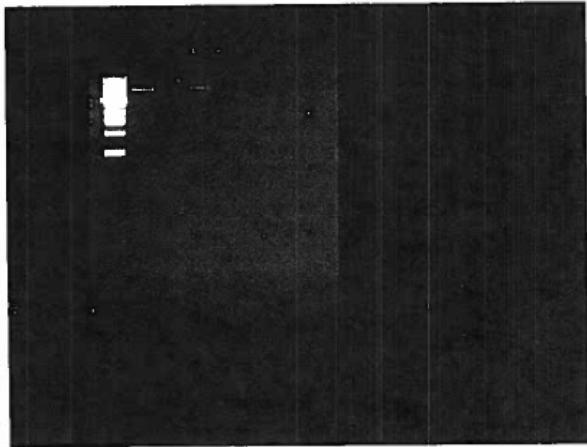


Figure 3. Electrophoresis gel of restriction enzymes. Lane 1 contains a 1kb ladder; Lane 2: SphI; Lane 3: XbaI; Lane 4: BamHI

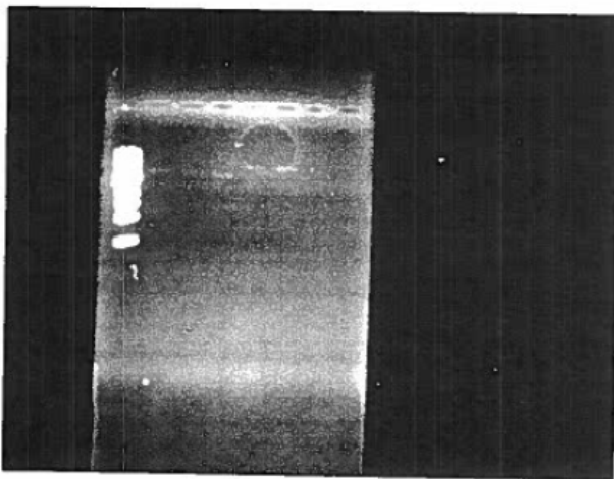


Figure 4. Electrophoresis gel of restriction enzymes. Lane 1 contains a 1kb ladder; Lane 2: NcoI; Lane 3: BspHI; Lane 4: BsoBI; Lane 5: HindIII; Lane 6: SmaI

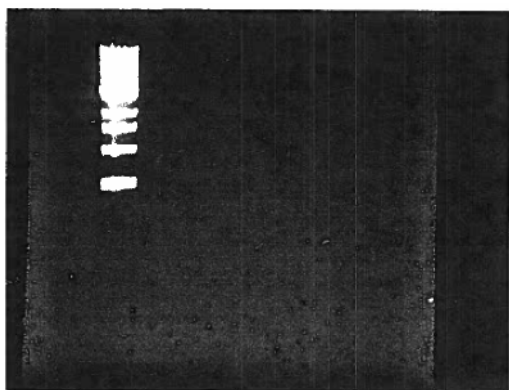


Figure 5. Electrophoresis gel of restriction enzymes. Lane 1 contains a 1kb ladder; Lane 2: *Xba*I

Eight different restriction enzymes were tested for restriction sites on the pCN51 shuttle vector. The enzymes chosen were due solely to availability in the laboratory. Only one tested enzyme, *Xba*I, had no cuts on the plasmid. All others (*Sph*I, *Hind*III, *Bam*HI, *Nco*I, *Bsp*HI, *Bso*BI, *Sma*I) appeared to have two cut sites. At this point, *Xba*I could potentially be used as the site to insert the desired *AdhE2* gene.

3.4 pCN51 Isolation

pCN51 was purchased as a frozen glycerol stock of *E. coli* containing the plasmid. It was necessary to run a QIAprep Spin Miniprep Kit to isolate the plasmid DNA. After the cells have been lysed with centrifugation, this kit provides a silica gel membrane that binds to the DNA with a high salt concentration, while allowing elution for DNA isolation in a lower salt concentration. Figure 6 shows the gel confirming isolation of the DNA.

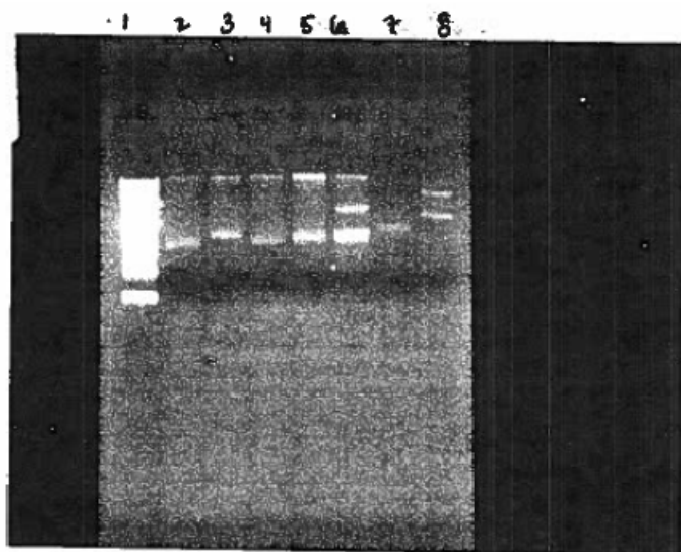


Figure 6. Electrophoresis gel of pCN51. Lane 1 contains a 1kb ladder. Lane 8 contains plasmid DNA. Lanes 2-7 do not pertain to this study.

After isolation from *E. coli*, amplification in *P. fluorescens* needed to be done in order to ensure the shuttle vector would be appropriate. As described in literature, pCN51 works with various strains, but *P. fluorescens* was never described. Transformation was successful, and plasmid still contained the kanamycin resistance.

Below are the results that confirmed the plasmid can be transformed into *P. fluorescens*.

Table 3

pCN51 transformation confirmation in <i>P. fluorescens</i>				
Tube	<i>Pseudomonas</i>	Kanamycin	pCN51	Growth
1	Yes	Yes	Yes	Yes
2	Yes	No	Yes	No
3	Yes	Yes	No	No
4	Yes	No	No	No

3.5 Green Fluorescent Protein

It is desirable that the new shuttle vector not only contain the addition of *AdhE2*, but also the green fluorescent protein (GFP). GFP is a protein that fluoresces a bright green color when it is exposed to blue light. With this gene also inserted into pCN51, it would make it easier to detect if the shuttle vector picked up or rejected the genetically engineered plasmid. The plasmid containing GFP, pGFPuv, is a 3.3kb plasmid (Fig 3) that has other desirable characteristics. The lac operon, located upstream of the 5' multiple cloning site, contains a mechanism that responds to the presence of glucose and lactose. When an inefficient amount of lactose is available, the lac repressor will halt the production of energy producing enzymes, acting as a regulating mechanism.

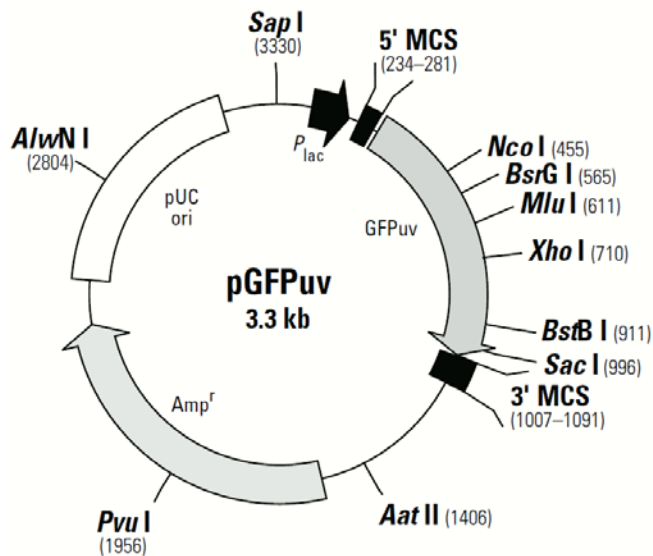


Figure 7. A plasmid map of pGFPuv showing the location of GFP and the lac operon (12).

The isolation of GFP from pGFPuv was successful using PCR. The electrophoresis gel in Figure 8 shows successful amplification of the GFP gene (1 kb PCR fragment).

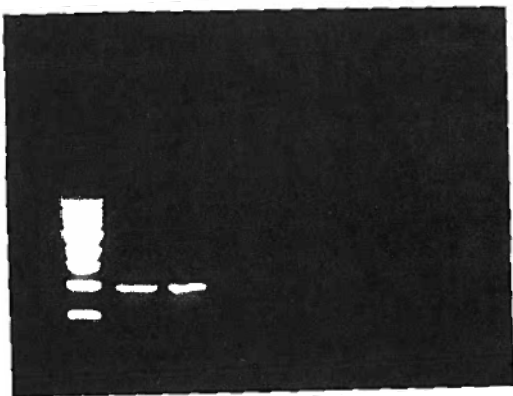


Figure 8. Electrophoresis gel of GFP. Lane 1: 1kb DNA ladder; Lane 2: GFP; Lane 3: GFP; Lane 4: control. Gel shows successful isolation of 1kb GFP protein.

Table 4 shows the primers designed that captured the 5' multiple cloning site, the lac operon, and the GFP protein. The total size of the PCR product was approximately 1 kb in length.

Table 4

Primers designed for GFP removal from pGFPuv	
<i>Forward Primer</i>	TCACTCATTAGGCACCCCAGGC
<i>Reverse Primer</i>	ACCGAAACGCGCGAGACGAA

3.6 Inserting GFP into pCN51

In order to successfully insert the PCR product, a restriction enzyme needed to be used that cuts at sites upstream of the lac operon ($\approx 100\text{bp}$) and downstream of the entire GFP protein ($\approx 1007\text{bp}$). We decided this could be done successfully in two different ways. The first option was to cut pCN51 with *Bam*HI (1 cut site) and fill in the sticky ends with Klenow and dNTP. The *AdhE2* gene could then be inserted in pCN51 with an appropriate restriction enzyme. While this would work, it would most likely be replicated with low frequency. The second option was to perform a limited digestion with restriction enzyme *Bam*HI; while *Bam*HI does cut in desired

sites, the largest DNA fragment of appropriate length would need to be isolated and then used for insertion of the *AdhE2* gene. Fig 4 shows the *Bam*HI cut sites on the DNA strand.

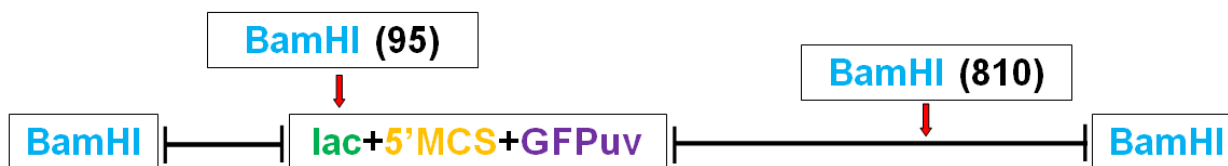


Figure 9. Desired DNA strand doing a limited digestion, showing enzyme restriction sites and the approximate location on the DNA strand.

3.7 Ongoing Work

Due to time constraints, our research team decided to stop working with the GFP gene and to insert the protein into pCN51 at a later date. The main focus of this project was to insert the *AdhE2* gene into pCN51. I am currently culturing *C. acetobutylicum*, and my next step is to remove the *AdhE2* gene from the bacterium. Table 4 shows the primers I will use for PCR.

Table 4	
Primers designed for AdhE2 removal from <i>C. acetobutylicum</i>	
<i>Forward Primer</i>	TGCTCATGACTTGCATGGAAAATTGGG
<i>Reverse Primer</i>	TCTCGACACGGACGCAA

To cut pCN51, the restriction enzyme *Bam*HI will be used (1 cut site), and then the plasmid will be treated with Mung Bean Nuclease from New England BioLabs. This will blunt end ligate the PCR fragment, allowing for easy insertion of the *AdhE2* gene. The gene can then be inserted with *Sph*I, a restriction enzyme that cuts pCN51 one time, and does not cut *AdhE2*. I will continue to work on this project throughout the beginning of the summer in order to complete the new *Pseudomonas*.

4. CONCLUSIONS

4.1 Summary

Through research and searching thorough literature, we have engineered a way to genetically alter *P. fluorescens* to allow it produce biobutanol. By using published articles and online databases, we were able to piece together a puzzle that allowed us to create a new process to ferment algae to make butanol. With the project being successful thus far, only a few steps remain before we will have created bacteria that could significantly alter the algal fermentation process. The need for alternative fuel is high, and research that drives butanol production via algae with new and improved methods is exactly what the algae/butanol fuel industry needs.

4.2 Future Work

After completion of the genetically engineered bacteria, it is necessary to confirm the new *Pseudomonas* is able to make butanol. This will be done using high performance liquid chromatography (HPLC). Algae will then be combined with the *Pseudomonas* to ensure that the *Pseudomonas* has not lost its lysing ability, and can still extract carbohydrates and sugars from the algae, along with it being able to produce biobutanol. The butanol production, along with the amount of sugars released, will be measured using HPLC and gas chromatography. The *Pseudomonas* will be checked for plasmid rejection. The butanol production process can then be optimized by altering the algae:*pseudomonas* ratio and fermentation times.

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